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The effects of hydrostatic pressure (1 atm to 408 atm) on the A_1 adenosine receptor-G_i protein-adenylate cyclase complex were studied in membrane preparations of two congeneric marine teleost fishes, Sebastolobus alascanus and S. altivelis. S. alascanus is common from 180-440 m; S. altivelis from 550-1300 m. Assay conditions for the binding of A_1 adenosine receptor agonists at 5°C and atmospheric pressure were optimized and the reaction conditions for the assay of adenylate cyclase were determined. The effects of hydrostatic pressure on basal adenylate cyclase activity and the response of adenylate cyclase to modulation by A_1 receptor occupancy were determined. Basal adenylate cyclase activity was inhibited 11 to 25% by 136 atm pressure and 29 to 41% by 408 atm. Inhibition by the A_1 adenosine receptor agonist cyclopentyladenosine (0.1 mM) was affected by pressure increases. [^{32}P]ADP-ribosylation of G protein substrates differed 10 fold between the Sebastolobus congeners and the extent of [^{32}P]ADP-ribosylation was altered by pressure increases. All of the components tested on this receptor-effector system (con't on back)

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19. Abstract continued
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PRINCIPAL INVESTIGATOR: Thomas F. Murray
      (In collaboration with Dr. Joseph F. Siebenaller Louisiana State
University)

CONTRACTOR: Oregon State University

CONTRACT TITLE: Effects of pressure on membrane-associated receptors and
      effector elements.

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INTRODUCTION

The objective of the first year of this study was to demonstrate the magnitude of the effects of hydrostatic pressure on the A_1 adenosine receptor - G_i protein - adenylate cyclase system.   To this end, we have set out to 1) optimize the conditions for the adenosine agonist binding assays; 2) optimize the conditions for adenylate cyclase assays; 3) examine ADP- ribosylation of G protein substrates by pertussis toxin under varied conditions, and 4) to survey these systems for susceptibility to perturbation by hydrostatic pressure. Experiments were designed to demonstrate the magnitude of any pressure effects and establish the suitability of this receptor-effector system as a model for studies of pressure effects on membrane signal transduction processes. We have focused our efforts on preparations from two congeneric marine teleost species which have similar life histories, experience similar temperatures, but differ in their depth distributions as adults.   These two species of the genus _Sebastolobus_ have served as a model system for studies of adaptation to hydrostatic pressures characteristic of the marine environment (e.g., Siebenaller and Somero, 1989).   Our results indicate that the A_1 adenosine receptor - G_i protein - adenylate cyclase system is sensitive to perturbation by hydrostatic pressures as low as 136 atm (the lowest pressure above 1 atm which we have tested to date), and that the magnitude and direction of the perturbation differs among species.

MATERIALS AND METHODS

Specimens. Demersal adult _Sebastolobus alascanus_ and _S. altivelis_ (Scorpaenidae) were collected by otter trawl at their depths of typical abundance by otter trawl off the coast of Oregon on cruises of the R/V _Wecoma_. Tissues were dissected and frozen in liquid nitrogen at sea and transported to the laboratory where the tissues were maintained at -80°C until used. _S. alascanus_ is common between 180 and 440 m; _S. altivelis_ is typically found between 550 and 1300 m.
      Additional experiments were performed with brain tissue from demersal adult

<u>Antimora rostrata</u> (Moridae) taken by otter trawl between 850 and 2500 m off the coast of Newfoundland, Canada. Rat brains were obtained from Pel Freez (Rogers, Ark).

   <u>Pressure apparatus.</u> The high pressure vessels, pump and guage are described in Hennessey and Siebenaller (1985) and are modeled after those described by Zobell and Oppenheimer (1950).

RESULTS

1) Conditions for adenosine agonist binding assays

   At atmospheric pressure, the effects of temperature on agonist binding to the A_1 adenosine receptor in brain tissue of 8 vertebrate species with body temperatures ranging from 1 to $40°C$ have been studied (Siebenaller and Murray, 1988). K_d values for the agonist [^{3}H]cyclohexyladenosine ([^{3}H]CHA) vary 30-fold among the species at a measurement temperature of $5°C$. At temperatures approximating the cell temperatures of the species, there is only a 4-fold range of values (Fig. 1). Binding entropies were positive for all species; values were largest for the warm-adapted species and smallest for the deep-living fishes. The density of receptors (B_{max}) was relatively insensitive to temperature variation (Fig. 2). In addition, the structure activity profiles of adenosine agonists in cold-adapted species (at a measurement temperature of $5°C$) are similar to the profiles obtained at higher temperatures (e.g., Siebenaller and Murray, 1986). Thus despite the perturbation by low temperatures of agonist binding to mammalian and avian A_1 adenosine receptors, agonist recognition and binding properties of the A_1 receptor have been retained in vertebrates adapted to body temperatures from 1 to $40°C$. These adaptive trends mirror those noted in prior studies of soluble enzyme homologs and muscle actins from species adapted to different temperatures (e.g., Siebenaller and Somero, 1989).

   In addition we have undertaken the characterization of binding of the antagonist 8-cyclopentyl-1,3[^{3}H]dipropylxanthine ([^{3}H]DPCPX) in cardiac membrane preparations from the <u>Sebastolobus</u> species. Based on the results summarized in Table 1, we have developed a routine method for the preparation of piscine cardiac membranes. Following homogenization in buffered 0.25M sucrose, the homogenates are centrifuged at 4,000 x g for 10 min. The resultant supernatant is then centrifuged at 48,000 x g for 20 min. The supernatant is then discarded and the pellet (P_2) resuspended in 25 mM imidazole (pH 7.2 at $22°C$) and again centrifuged at 48,000 x g for 20 min. This pellet is resuspended in 25 mM imidazole and used directly in radioligand binding assays. This membrane preparation exhibits a 3 to 4- fold enrichment in [^{3}H]DPCPX binding sites as compared to the crude homogenate. As a consequence of the relative enrichment in A_1 adenosine receptor sites, this membrane preparation provides an adequate signal to noise ratio for [^{3}H]DPCPX specific binding. The density of [^{3}H]DPCPX binding sites in these membranes is comparable to that observed in porcine and chicken atrial membranes (Leid et al., 1988; Blair et al., 1989). This preparation will therefore allow an assessment of the influence of pressure on A_1 adenosine receptor - G protein coupling in <u>Sebastolobus</u> cardiac membranes. This will permit us to determine whether pressure related differences in agonist binding to brain A_1 receptors generalize to heart receptors. Given the significance of endogenous adenosine as a physiological regulator of cardiac function (e.g., Murray et al., 1989), the results of these studies may have important implications regarding the cardiovascular sequelae of deep diving.

Table 1. Distribution of [³H]DPCPX binding sites in piscine cardiac membrane preparations.

Preparation[a]	[³H]DPCPX BOUND (fmol/mg protein)
crude homogenate	1.9
P_1 (4,000 x g)	1.2
P_2 (10,000 x g)	4.0
P_3 (48,000 x g)	7.5

[a] Whole heart homogenized in 0.25 M sucrose containing 10 mM EDTA and 10 mM Tris-HCl, pH 7.6 at 22°C, using a Dounce homogenizer.

2) Conditions for adenylate cyclase assays

We have performed a series of experiments at atmospheric pressure and 5°C to determine optimal concentrations of the components in our assay of adenylate cyclase. Specifically, we have determined the concentrations of membrane protein, guanosine triphosphate (GTP), magnesium and sodium chloride, both with and without the adenylate cyclase-activating diterpene forskolin which yield maximal reaction velocities and sensitivity to A_1 adenosine agonists (e.g., Fig. 3). Typical reaction conditions are described in the legend to Fig. 4 which illustrates a time course of the adenylate cyclase reaction in brain membrane preparations from the Sebastolobus species. Using our reaction mixture at 5°C, the reaction velocity is linear with respect to time for at least 180 min.

3) ADP-ribosylation of G protein substrates by pertussis toxin

Guanine nucleotide binding proteins function as signal transducers in plasma membranes, coupling receptor occupancy to adenylate cyclase activity. The alpha subunit of the inhibitory G protein (G_i) is specifically ADP- ribosylated by pertussis toxin (islet activating protein). The petussis toxin substrate in S. altivelis brain membranes is approximately 10-fold more susceptible to to [³²P]ADP ribosylation than that of S. alascanus (Fig. 5). This increased labeling in S. altivelis membranes could be due to the conformation of G_i, an increased amount of the alpha subunit, the intrinsinc structure of the alpha subunits, or the availability of the beta-gamma complex of the G_i heterotrimer within the membrane.

4) Survey of the effects of hydrostatic pressure on the A_1 adenosine receptor - G_i protein - adenylate cyclase system

For incubations at elevated pressures, samples are transferred to polyethylene tubing. The tubing is then trimmed to exclude air bubbles and sealed using a pipet heat sealer. For the adenylate cyclase assays we use [³H]cAMP as an internal standard to monitor the recovery of [³²P]cAMP through the sealing and incubation and through the column chromatography steps isolating the [³²P]-labeled cAMP from the [³²P]ATP following Salomon et al. (1974). Samples are incubated in high pressure vessels which are maintained in a refrigerated circulating water bath. Incubations are for 120 min or more so that the approximately 2-4 min required to seal and pressurize a group of 4 samples and the 2-4 min to remove the samples are not a substantial fraction of the entire

incubation time. Samples sealed and incubated at atmospheric pressure have adenylate cyclase activities identical to samples which are simply incubated in test tubes. The pressure of the incubation vessel is tested prior to termination of the assays.

We have used this system to assay hydrostatic pressure effects on A_1 receptor - sensitive adenylate cyclase activity and on ADP-ribosylation of G proteins. Thus far we have employed hydrostatic pressures of 1, 136, 272 and 408 atm. These pressures were chosen either to approximate the pressures experienced by the species in situ (see below), or in the case of the highest pressure chosen, to magnify any pressure sensitivity. All of the elevated pressures we have employed have affected the A_1 receptor - adenylate cyclase system.

Our pressure studies to date have included three teleost fish species, Sebastolobus alascanus (Scorpaenidae) (common from 180-330 m); S. altivelis (Scorpaenidae) (440-1300 m); and Antimora rostrata (850-2500 m). We have also begun studies using membrane preparations from rat (Rattus rattus) brain.

Our initial pressure experiments have focused on (1) the effect of hydrostatic pressure on basal adenylate cyclase activity and (2) the effects of pressure on the pattern of inhibition of adenylate cyclase by the A_1 receptor agonist cyclopentyladenosine (CPA). For both of the species of Sebastolobus all of the elevated pressures tested inhibited basal adenylate cyclase activity. Adenylate cyclase activity was inhibited 11 to 25% by 136 atm of pressure, and 29 to 41% by 408 atm pressure. The degree of inhibition by pressure was dependent on the ionic strength of the assay medium. Under low ionic strength conditions, the apparent volume changes associated with the pressure-inhibition of adenylate cyclase activity average 23 and 34 ml mol^{-1} for S. alascanus and S. altivelis, respectively. Results of individual experiments are illustrated in Fig. 6. In contrast, the adenylate cyclase from the deep-living fish, Antimora rostrata, assayed under identical conditions is unaffected by pressures up to 272 atm, the highest pressure tested to date with this species. The apparent volume change for the adenylate cyclase reaction of this deeper living species is 0 ml mol^{-1}.

At atmospheric pressure, 0.100 mM CPA substantially inhibits adenylate cyclase activity (see Fig. 6). For S. altivelis, 408 atm pressure appears to reverse this inhibition. In contrast, for S. alascanus, increased pressures to 408 atm do not appear to affect the pattern of CPA inhibition of adenylate cyclase (Fig. 6). The mechanisms underlying this differential effect of hydrostatic pressure on CPA inhibition remain to be elucidated, and our future effects will address this. The possibility that the differences between the two species are due to an enhancement of the A_2 adenosine receptor or an enhancement of the efficacy of G_s - adenylate cyclase interaction in S. altivelis will be investigated in further studies. In experiments with the deep-living A. rostrata, the agonists CPA and 5'-N- ethylcarboxamidoadenosine (NECA) were tested at atmospheric pressure and 272 atm pressure. Increased pressure either had no effect, or increased the efficacy of the agonists in inhibiting adenylate cyclase activity, again contrasting with the patterns observed for the Sebastolobus species.

We have initiated experiments testing the effects of hydrostatic pressure on adenylate cyclase activity in rat brain tissue using the protocol developed for piscine adenylate cyclase. At 5°C, hydrostatic pressure inhibits basal adenylate cyclase activity to a much greater extent than that observed with the fish membrane preparations. Application of 240 atmospheres of pressure causes a 55.6% ± 2.6% inhibition relative to the activity at atmospheric pressure (4 experiments);   340 atm inhibits basal activity 65.9% ± 4.7% relative to

atmospheric pressure (3 experiments). Under conditions of elevated pressure, the adenylate cyclase retains sensitivity to modulation by CPA. We are planning experiments to determine whether such dramatic inhibition of adenylate cyclase is apparent at lower pressures, in the range of 10 to 100 atm, and to determine the influence of temperature on this inhibition.

The effect of 408 atm on pertussis toxin catalyzed [^{32}P]ADP- ribosylation of the alpha subunit of G_i in the Sebastolobus species was investigated. At atmospheric pressure membrane preparations from S. altivelis incorporate 10 to 15 times more label per unit membrane protein than do preparations from S. alascanus (Fig. 5). This pattern is dramatically altered by incubation at 408 atm pressure. There is a slight decrease in labeling of the S. altivelis preparation relative to the 1 atm results. At 408 atm the labeling of the S. alascanus preparation increases approximately 10 to 20-fold, to a level comparable to that of the S. altivelis membrane preparations. Interestingly, there appears to be a suggestion of ADP-ribosylation in the absence of pertussis toxin catalyst at 408 atm (Fig. 5). Further studies of this dramatic effect of pressure on the susceptibility of G protein alpha subunits to ADP-ribosylation are planned.

CONCLUSIONS

Our initial studies of the effects of elevated hydrostatic pressure on the A_1 adenosine receptor - G_i protein - adenylate cyclase complex in central nervous tissue indicate that the components of this system are markedly susceptible to perturbation by pressure increases, and the magnitude and direction of the perturbations differ among species.

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FIGURE LEGENDS

Figure 1. The effects of temperature on K_d of [^{3}H]CHA. Filled square: Rattus rattus; open square: Gallus domesticus; open inverse triangle: Epinephelus fulvus; open triangle: Sebastolobus alascanus; open circle: Macrourus berglax; filled triangle: S. altivelis; filled circle: Coryphaenoides rupestris; filled inverse triangle: Antimora rostrata.
[From Siebenaller and Murray, 1988]

Figure 2. Effects of temperature and 5 mM $MgCl_2$ on B_{max} values of [^{3}H]CHA for Coryphaenoides rupestris, Macrourus berglax and Gallus domesticus brain membranes. Open bars: no added $MgCl_2$; filled bars: 5 mM $MgCl_2$. Means of at least two experiments are shown; the standard error for each individual estimate of B_{max} is less than 9%; replicate estimates of B_{max} differ by less than 10%.
[From Siebenaller and Murray, 1988]

Figure 3. Concentration response curve of GTP dependence of the CPA inhibition of adenylate cyclase. Incubations were at 5°C. Incubation conditions are as described in the legend to Fig. 4. A. Sebastolobus alascanus B. Sebastolobus altivelis.

Figure 4. Time course of the adenylate cyclase reaction at 5°C with brain membranes from Sebastolobus alascanus and S. altivelis. Membranes were prepared by homogenization with a Dounce in 100 volumes of 10 mM HEPES, pH 7.6 at 5°C, 1 mM dithiothreitol. The homogenate was centrifuged 27,000 x g for 10 min, the pellet resuspended in 100 volumes and centrifuged. The pellet was resuspended and brought to 7.5 international units adenosine deaminase ml^{-1} and incubated at 18°C for 30 min. The mixture was chilled on ice, centrifuged and the pellet resuspended for use in the assays. The incubation mixtures contained in a volume of 0.15 ml: 51 mM HEPES, pH 7.6 at 5°C, 100 mM NaCl, 3.4 mM magnesium acetate, 0.20 mM papaverine, 0.10 mM cAMP, 0.5 mM 2-deoxy ATP, 0.01 mM GTP, 7.5 international units of adenosine deaminase ml^{-1}, 1.3 mg ml^{-1} phosphocreatine, 0.7 mg ml^{-1} creatine phosphokinase, 1.1 mg ml^{-1} Bacitracin, 0.1 mg ml^{-1} soybean trypsin inhibitor and approximately 10,000 cpm [^{3}H]cAMP per tube.

Figure 5. Autoradiogram of pertussis toxin catalyzed [^{32}P]ADP ribosylation of G protein substrates in Sebastolobus brain membrane preparations. Samples were prepared in 100 mM Tris-HCl, pH 7.6 at 5°C, 0.3 mg ml^{-1} soybean trypsin inhibitor, 3.4 mg ml^{-1} Bacitracin. Incubations were at 5°C, at 1 or 408 atm. Final concentrations of constituents were: 100 mM Tris-HCl, pH 7.5 at 5°C, 25 mM dithiothreitol, 0.1 mM GTP, 2 ug activated pertussis toxin, 25 to 50 ug membrane protein and 5 uCi (approximately 10 uM) [^{32}P]NAD.
Lane 1: Sebastolobus altivelis, minus pertussis toxin (PT), 408 atm.
Lane 2: S. altivelis, plus PT, 408 atm.
Lane 3: H$_2$O control, no tissue, plus PT, 408 atm.
Lane 4: S. alascanus minus PT, 408 atm.
Lane 5: S. alascanus plus PT, 408 atm.
Lane 6: S. altivelis plus PT, 1 atm.
Lane 7: S. altivelis minus PT, 1 atm.
Lane 8: H$_2$O control, no tissue, plus PT, 1 atm.
Lane 9: S. alascanus, minus PT, 1 atm.
Lane 10: S. alascanus, plus PT, 1 atm..

Note that in this xerox reproduction of the autoradiogram that the faintly labeled 41,000 molecular weight band in lane 10 is not apparent. The 41,000 molecular weight band in lane 2 was reproduced somewhat better.

Figure 6. The effects of hydrostatic pressure on basal adenylate cyclase activity (-CPA) and A$_1$ adenosine receptor inhibited adenylate cyclase activity (+CPA). The concentration of CPA used was 0.100 mM. A. Sebastolobus alascanus. B. S. altivelis. Incubation conditions as described in the legend of Fig. 4.
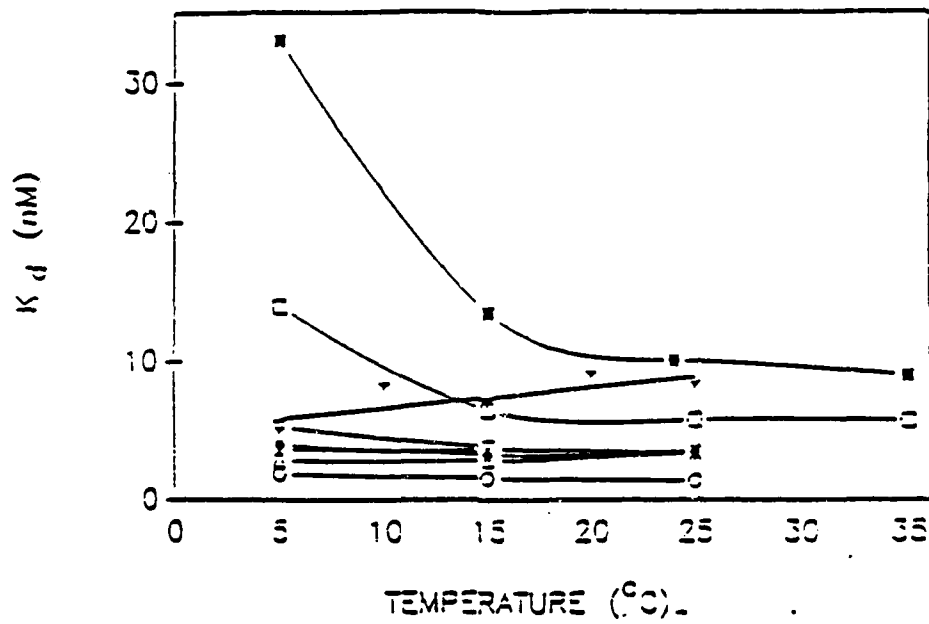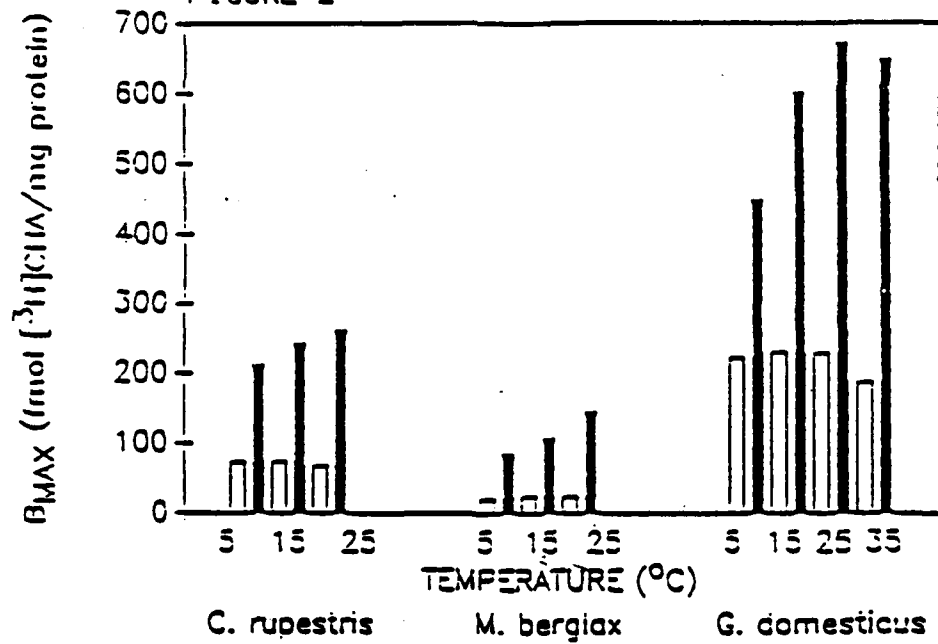
FIGURE 1

FIGURE 2

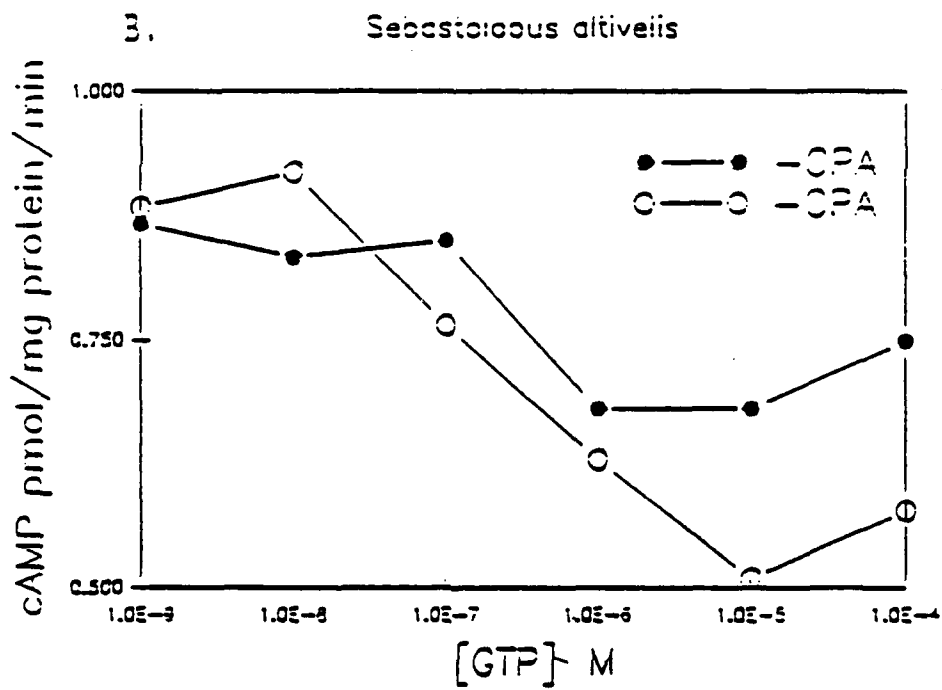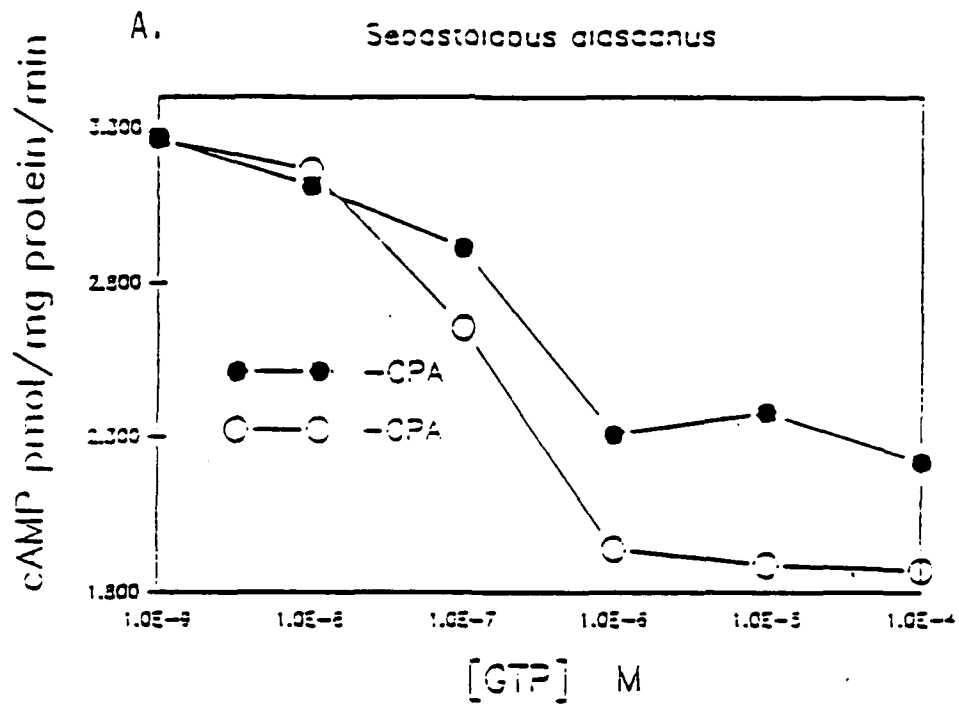C. rupestris   M. bergiax   G. domesticus

FIGURE 3

A. Sebastolcous diascanus

B. Sebastolcous altiveiis

FIGURE 4

FIGURE 5

LANE

1 2 3 4 5 6 7 8 9 10

ORIGIN
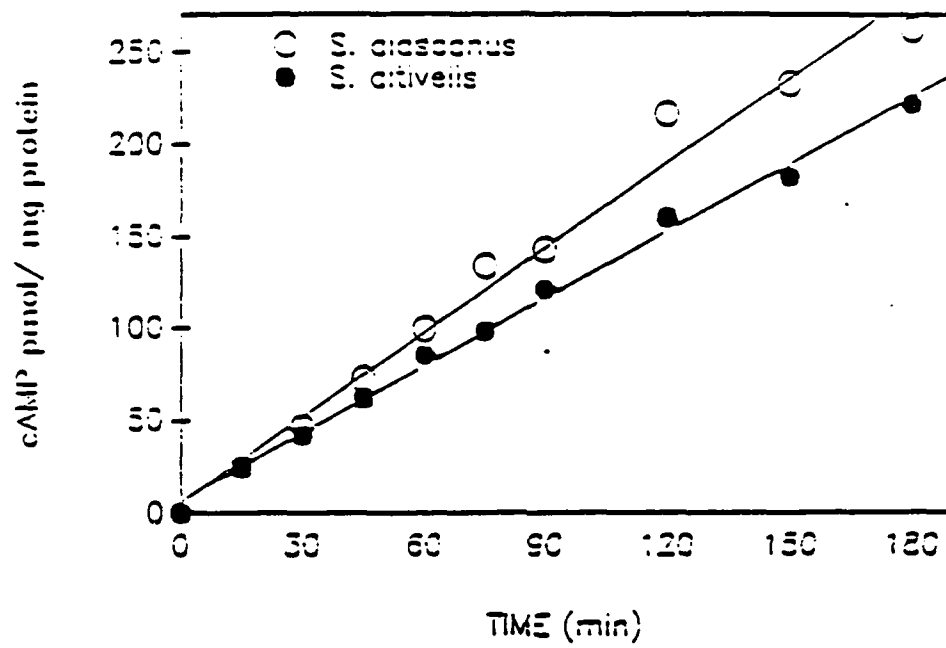
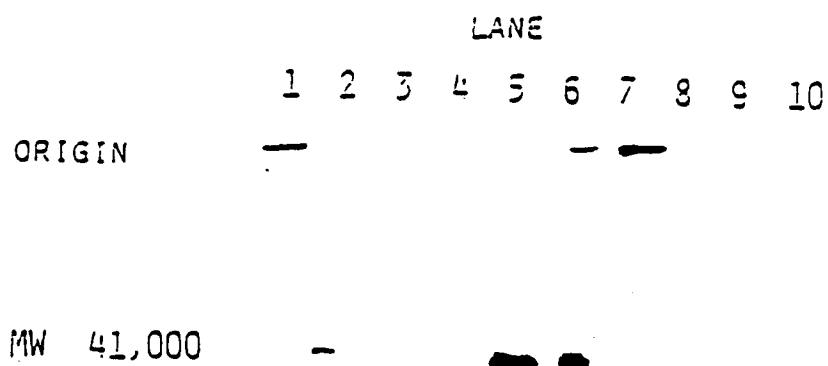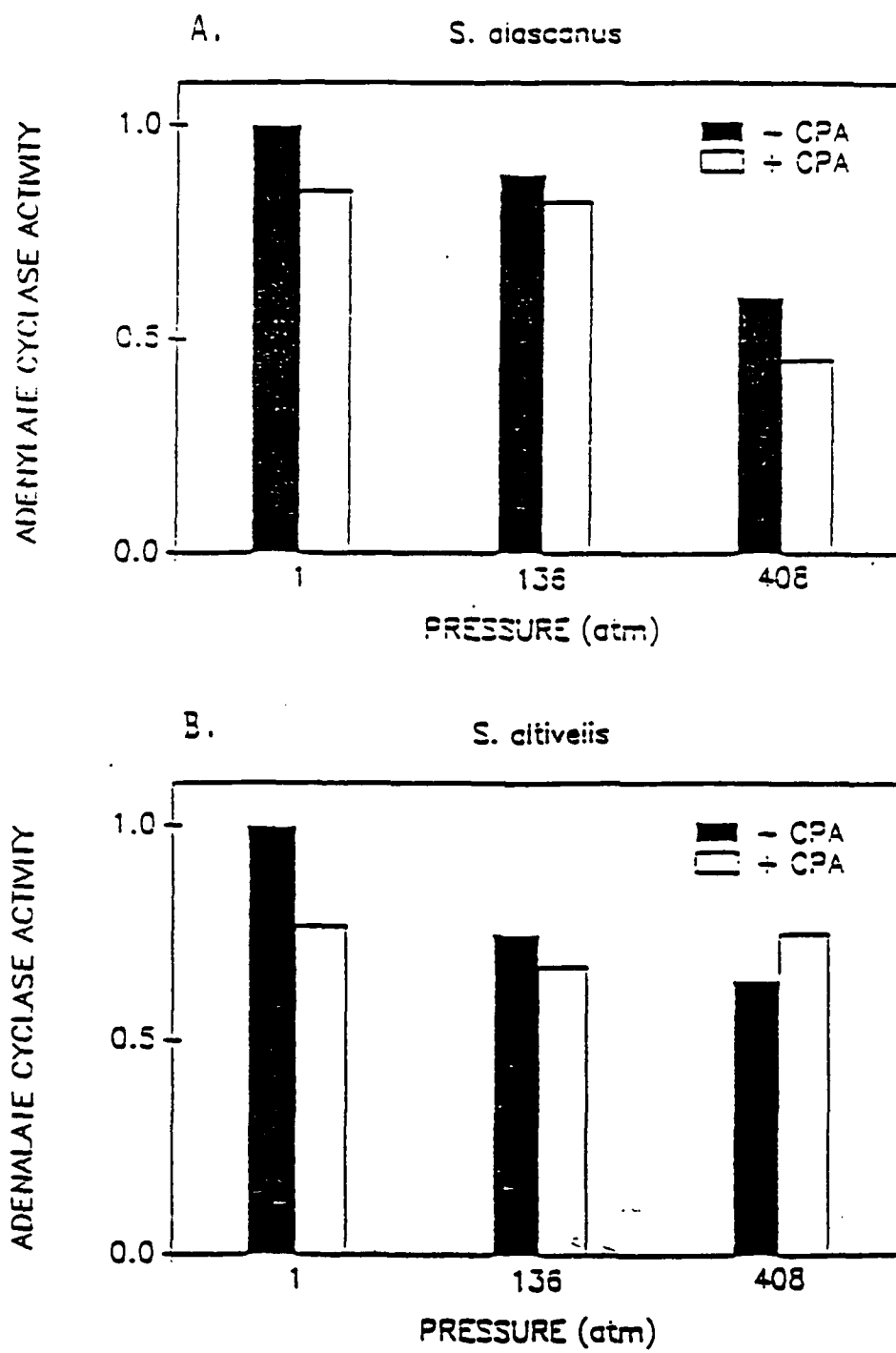MW 41,000

FIGURE 6

A.   S. diasconus

B.   S. altivelis

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